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Comparison of in vitro estrogenic activity and estrogen concentrations in source and treated waters from 25 U.S. drinking water treatment plants

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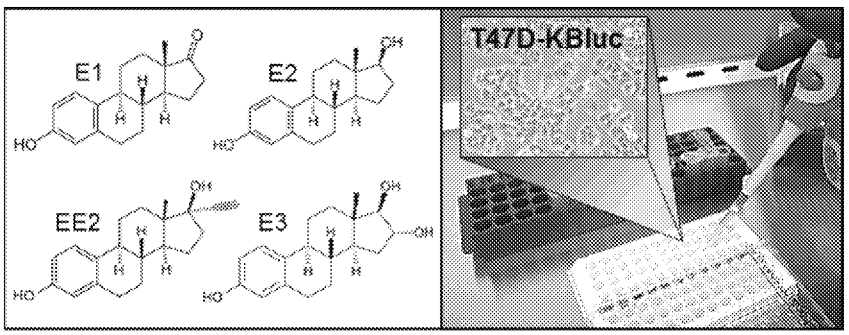
HIGHLIGHTS

- Screen of U.S. source and treated water for estrogens and estrogenic activity.
- T47D-KBluc bioassay reporting limits were below LC-FTMS method quantitation limits.
- Drinking water treatment processes effectively reduced estrogenicity.
- All samples were below estimated trigger values for adverse effects.
- In vitro bioassay was an effective complement to targeted chemical analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

In vitro bioassays have been successfully used to screen for estrogenic activity in wastewater and surface water, however, few have been applied to treated drinking water. Here, extracts of source and treated water samples were assayed for estrogenic activity using T47D-KBluc cells and analyzed by liquid chromatography-Fourier transform mass spectrometry (LC-FTMS) for natural and synthetic estrogens (including estrone, 17β-estradiol, estriol, and ethinyl estradiol). None of the estrogens were detected above the LC-FTMS quantification limits in treated samples and only 5 source waters had quantifiable concentrations of estrone, whereas 3 treated samples and 16 source samples displayed in vitro estrogenicity. Estrone accounted for the majority of estrogenic activity in respective samples, however the remaining samples that displayed estrogenic activity had no quantitative detections of known estrogenic compounds by chemical analyses. Source water estrogenicity (max, 0.47 ng 17β-estradiol equivalents (E2Eq) L^{-1}) was below levels that have been linked to adverse effects in fish and other aquatic organisms. Treated water estrogenicity (max, 0.078 ng E2Eq L^{-1}) was considerably below levels that are expected to be biologically relevant to human consumers. Overall, the advantage of using in vitro techniques in addition to analytical chemical determinations was displayed by the sensitivity of the T47D-KBluc bioassay, coupled with the ability to measure cumulative effects of mixtures, specifically when unknown chemicals may be present.

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1. Introduction

The presence of endocrine disrupting compounds (EDCs) in surface water has been a concern for decades (Guillette et al., 1995). EDCs primarily consist of synthetic organic compounds used in select pharmaceuticals, personal care products, pesticides, and industrial-use chemicals (e.g., plasticizers, flame retardants) (NIEHS, 2014). Once these compounds enter surface waters they can pose a risk to aquatic organisms, which may be adversely affected by physiological perturbations that can lead to altered development and/or reproductive potential (Jobling et al., 1998; Kidd et al., 2007). Additionally, there has been growing concern that EDCs may pose a risk to human health from potential consumption of aquatic organisms (Sjodin et al., 2000) and/or drinking water (Benotti et al., 2009; Caldwell et al., 2010) contaminated with EDC residues.

In addition to synthetic compounds, the list of environmental EDCs includes natural estrogens and androgens. These compounds include both animal steroids (largely of mammalian and avian origin) and plant-based sterols and stanols, which enter surface water via human wastewater treatment effluent (Belfroid et al., 1999), agricultural livestock waste runoff (Yost et al., 2013), and pulp and paper mill effluent (Jenkins et al., 2001). Natural estrogens, as well as the synthetic estrogen 17α-ethinyl estradiol (EE2) in particular, are of concern due to their potency at low concentrations (e.g., ~ng L^{-1} range) and potential for exposures to humans and wildlife (Sumpter, 2014). Estrone (E1), 17β-estradiol (E2), and estriol (E3) have been identified in wastewater treatment plant effluent and surface waters (Alvarez et al., 2013; Kolpin et al., 2002). These compounds may occur individually, however they typically co-occur with other EDCs in complex mixtures leading to possible exposure to a wide range of natural and synthetic EDCs.

Once EDCs enter surface waters, they are subject to both dilution and attenuation from environmental processes (e.g., hydrolysis, photolysis, microbial degradation, sorption). However, the distance between wastewater effluent discharge points and drinking water treatment plant (DWTP) intake points has been decreasing as both the demand for treated water and volume of sewage to be treated become greater (NRC, 1998). As such, the potential for environmental attenuation by dilution and degradation decreases and the likelihood of biologically-relevant levels of EDCs entering drinking water treatment facilities increases. Further, the broad range of drinking water treatment processes employed have variable efficacy for attenuating the concentrations of EDCs that may be present in DWTP source water (Snyder et al., 2003). Depending on the concentrations of EDCs in the source water and the treatment plant efficacy, EDC residues may persist into drinking water (Benotti et al., 2009; Kuch and Ballschmiter, 2001; Westerhoff et al., 2005).

The presence of EDCs in water samples has traditionally been detected and quantified using analytical chemistry. While analytical determinations remain essential to understanding sample chemical composition and identification of specific causative agents, there are drawbacks including limits of instrumental sensitivity and limits on the number of compounds that can be included in a given analytical method. Problematically, some EDCs may be biologically active at concentrations below analytical detection limits and many more compounds may be present than those available in a given analytical method. Bioanalytical tools, such as in vitro assays, have been utilized recently to assess the biological activity of environmental samples that may contain unknown chemical compounds and/or mixtures of compounds below analytical detection limits (Escher et al., 2014). In vitro assays have been developed for assessing the ability of a given sample to interact with the estrogen (Wilson et al., 2004) and androgen (Wilson et al., 2002) receptors, as well as others. Compared to analytical methods, in vitro assays are typically less expensive and allow for medium to high throughput screening of samples for a variety of hormonal activities, providing a cumulative measure of both exposure and biological effect.

This paper is one of a series describing a comprehensive study on the presence, concentrations, and persistence of contaminants of emerging concern (CECs) in source and treated waters of the United States. This was a joint effort of the U.S. Environmental Protection Agency (USEPA) and the U.S. Geological Survey (USGS), as part of a long-term interagency agreement. A primary goal of this study was to provide accurate, objective information regarding the potential for human exposure to biologically active/toxicologically-relevant concentrations of CECs via treated water. A secondary goal was to evaluate removal, if any, of CECs from source waters by current use drinking water treatment processes under typical plant operating conditions.

The USEPA/USGS collaboration examining CECs in source and treated water was conducted in two phases. Phase I examined 9 DWTPs for 64 chemical analytes. In Phase II the effort was expanded to include sample collection from 25 DWTPs located in 24 different states with analysis of 233 chemical analytes and 14 microbial pathogens. The goal was to sample DWTPs that utilize a variety of disinfection techniques and treat a broad range of production volumes, but with priority towards plants located downstream of known wastewater outfalls (i.e., those most likely to contain source water contamination in order to define the upper boundaries of CEC concentrations). Additionally, Phase II included an in vitro bioassay in order to provide a more comprehensive risk assessment of estrogens/estrogenicity associated with treated water. Our effort in the collaboration, described herein, was to assess the estrogenicity of both source and treated water collected from DWTPs across the United States. Each sample was assessed using the T47D-KBluc in vitro assay and concentrations of natural estrogens (E1, E2, E3) and the synthetic estrogen, EE2, were quantified using analytical approaches.

2. Materials and methods

2.1. Chemistry

2.1.1. Reagents and standards

Solid anhydrous analytical standards of E1 (purity, 100.8%; lot, B0294; CAS# 53-16-7), E2 (purity, 100.6%; lot, H733; CAS# 50-28-2), EE2 (purity, 98.3%; lot, H923; CAS# 57-63-6), and E3 (purity, 98.5%; lot, B0173; CAS# 50-27-1) were purchased from Steraloids (Wilton, NH). Methanol, acetone, and hexane used in sample preparation were Optima grade and purchased from Fisher Scientific (Pittsburgh, PA). Reagent grade water was purified to 18 MΩ cm^{-1} using MilliPore MilliQ (Billerica, MA) followed by EDSPak (Millipore) carbon filtration. Dansyl chloride was purchased from Sigma-Aldrich (St. Louis, MO). The stably labeled isotopic analogues $^{13}C_6$-E2, $^{13}C_6$-E1, and $^{13}C_2$-EE2 were purchased from Cambridge Isotopes Laboratory (Tewksbury, MA) and d_3-E3, d_8-Bisphenol A, and d_{16}-Bisphenol A were purchased from CDN Isotopes (Pointe-Claire, Canada). Methanol and acetonitrile used in chromatographic separation were Chromasolve Plus grade and purchased from Sigma-Aldrich. Puriss grade formic acid modifier was purchased from Fluka (St. Gallen, Switzerland).

2.1.2. Sample collection and preservation

Source and treated water samples were collected from 25 drinking water treatment plants from across the U.S. (details in Supplemental Table S1) in 600 mL silanized glass bottles containing 6 mg $CuSO_4 \cdot 5H_2O$ as a preservative and 6 mg ascorbic acid as a dechlorination agent. Treated samples consisted of 1.2 L (two 0.6 L sample bottles) while source samples were either 1.2 L (DWTP 3, 4, 5, 12, 13, 14, 21, 22, 23, 26, 27, 28, 29) or 0.6 L (DWTP 1, 2, 10, 11, 15, 16, 17, 18, 19, 20, 24, 25) depending on sample turbidity (i.e., smaller volume used for samples with high turbidity). Source and treated water samples were collected with DWTP hydraulic residence time accounted for such that the treated sample was comparable to the source sample (i.e., approximately same parcel of water entering DWTP as that exiting the plant). Following collection, samples were transported on ice, stored

at 4 °C, and extracted within 72 h of collection. Primary, duplicate, and matrix spikes (lab fortified with 1 ng of each estrogen prior to extraction) for source and treated samples were collected at every site.

2.1.3. Solid phase extraction and derivatization

Prior to extraction, Na_2EDTA (24 mg per 600 mL) was added to samples to reduce the concentration of copper in the extract and d_{16}-Bisphenol A (5 ng) was added as an extraction surrogate. Samples were passed through glass fiber filters (Millipore APFB; 1 μm) on top of 47 mm C_{18} Empore disks (3 M, St. Paul, MN). Disks and filters were rinsed with 20 mL 20% (v:v) methanol in water then eluted with 9 mL 10% acetone in methanol. One-sixth of the extract volume was removed for in vitro bioassay and concentrated to dryness. This aliquot represented either 100 mL or 200 mL equivalents of the original sample. The portion of extract to be analyzed by LC-FTMS was then spiked with a solution containing 1 ng of each of the stable isotope internal standards ($^{13}C_6$-E1, $^{13}C_6$-E2, $^{13}C_2$-EE2, d_3-E3, and d_8-BPA) and concentrated to dryness.

Analytical sensitivity was further improved by reaction of the phenolic moiety of each target compound with dansyl chloride. Briefly, the dry extract residue was resuspended in 0.1 mL $NaHCO_3$ (0.1 M, aqueous) followed by reaction with 0.1 mL dansyl chloride (1 mg mL^{-1} in acetone) for 5 min at 70 °C. As a cleanup step, the solution was extracted (liquid-liquid) with three 0.5 mL aliquots of hexane. The organic layers were combined, concentrated to dryness and stored at −20 °C until analysis.

2.1.4. Determination by LC-FTMS

Dry extracts were resuspended in 50:50 methanol:water. The analytes were separated using an Acquity ultra performance liquid chromatograph (UPLC) with a Bridged Ethylene Hybrid (BEH) Phenyl column (1.7 μm, particle size; 2.1 mm × 50 cm; Waters Corp., Milford, MA) and a separation gradient consisting of 0.02% formic acid, methanol, and acetonitrile. Identification and quantification of individual compounds was performed using a Thermo Scientific LTQ Orbitrap Discovery (Hybrid Ion Trap-Orbitrap Fourier Transform Mass Spectrometer; Waltham, MA) operated in selective reaction monitoring (SRM) mode. An electrospray ionization source, operated in positive ion mode, was used to introduce the sample to the mass spectrometer. Identification of the target analytes was performed in high-resolution by monitoring a quantitation daughter ion and two confirmatory daughter ions. Identification of the internal standards was performed using a quantitation ion and a single confirmation ion. LC-FTMS specifications for each target and quality control compound are listed in Table 1. Quantitation was performed using a seven point calibration curve for each compound and matrix effects were compensated for by using isotope dilution (ratio of analyte:internal standard peak areas).

2.1.5. Analytical QA/QC

Reagent blanks, field blanks, and positive control samples (lab fortified reagent water) were included in each extraction batch. Extraction recovery of 50–150% was considered acceptable for all fortified controls and the extraction surrogate (BPA-d_{16}). Only concentrations that were above the lowest concentration minimum reporting limit (LCMRL) are reported (USEPA, 2010). The LCMRL is the lowest spiking concentration such that the probability of spike recovery in the 50% to 150% range is at least 99%. The LCMRL values for E1, E2, EE2, and E3 were 0.09, 0.1, 0.4, and 0.09 ng L^{-1}, respectively. Details on sample collection and additional chemical analyses can be found in the overview manuscript by Glassmeyer et al. (2017).

2.2. In vitro assay

2.2.1. Chemicals and sample preparation

E2 (purity, 98%; lot, 28H0818; Sigma Aldrich) served as reference estrogen and ICI 182,780 (purity, 99%; batch, 20A/116982; CAS# 129453-61-8; ICI Pharmaceuticals, United Kingdom) served as anti-estrogen control (i.e., inhibitor). Extracts, prepared as described above, were blown to dryness under nitrogen and then resuspended in 50 μL ethanol (EtOH, 100%; Pharmco-AAPER, Brookfield, CT) prior to being diluted in cell culture medium for use in the bioassay.

2.2.2. Transcriptional activation assay

The T47D-KBluc assay was developed by USEPA (Wilson et al., 2004) for screening chemicals and mixtures for estrogen receptor agonism and antagonism and the cell line has been deposited at American Type Culture Collection (ATCC) for use by other investigators (#CRL-2865; Manassas, VA). T47D-KBluc cells naturally express endogenous estrogen receptors and were engineered to stably express a triplet estrogen response element-luciferase promoter-reporter gene construct. Cells were maintained and assayed as described by Wilson et al. (2004) with minor modifications as described herein. Briefly, T47D-KBluc cells were maintained in RPMI (phenol red-free; Gibco, Grand Island, NY) growth medium supplemented with 10% fetal bovine serum (FBS; characterized, Hyclone, Logan, UT), 2.5 g L^{-1} glucose, 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g L^{-1} $NaHCO_3$, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 0.25 μg mL^{-1} amphotericin B. One week prior to testing samples, maintenance media was replaced with antibiotic-free media containing 10% dextran coated charcoal stripped FBS (DCC-FBS, Hyclone) to minimize residual estrogens from the cultures. Mid-week cells were fed with fresh 10% DCC-FBS media. At the end of the week (day 7) cells were harvested from flasks and seeded in 96-well plates (1×10^4 cells $well^{-1}$ in 100 μL 5% DCC-FBS; Costar 3610, Corning Inc., Corning, NY). The following day (day 8), cell culture media was replaced with sample extracts serially diluted in 5% DCC-FBS media (≤0.2% final ethanol concentration). The initial sample extract dilution was 1:1000 into media followed by 2-fold serial dilutions in media down to a 1:128,000 dilution. A standard curve of E2 (0.3, 1, 3, 10, 30 pM), vehicle blank (EtOH), inhibitor, and E2 plus inhibitor were run concurrently with the sample on each plate. Each sample was tested in a minimum of two separate experiments on 96 well plates with 4 replicate wells per dilution level on each plate. After a 24 h incubation,

Table 1
LC-FTMS parameters for the detection and quantitation of target compounds and isotopically-labeled internal standards in extracts of source and treated water samples from DWTPs.

Compound	Retention time (min)	Parent mass (m/z)	Quantitation mass (m/z)	Collision energy (%)	Confirmation mass (m/z)	Second confirmation mass (m/z)
Dansyl estriol	9.3	522.231	458.268	30	443.245	171.104
d_3-Dansyl estriol	9.3	525.3	461.0	30	446.3	–
Dansyl estradiol	11.7	506.236	442.273	25	427.250	171.104
$^{13}C_6$-dansyl estradiol	11.7	512.3	448.3	25	433.3	–
Dansyl ethinylestradiol	12.0	530.336	466.273	27	451.250	171.104
$^{13}C_2$-dansyl ethinylestradiol	12.0	532.3	468.3	27	453.2	–
Dansyl estrone	12.3	504.220	440.260	27	425.234	171.104
$^{13}C_6$-dansyl estrone	12.3	510.2	446.3	27	431.3	–
d_8-Didansyl bisphenol A	14.2	703.3	469.2	27	371.8	–
d_{16}-Didansyl bisphenol A	14.1	709.2	475.6	27	377.6	–

luciferase activity was quantified in relative luminescence units (RLU) using a luminometer (BMG Fluostar Omega; BMG Labteck, Durham, NC). Throughout this process cells were visually examined for any signs of cytotoxicity. If cytotoxic effects were observed in a given well, data from that well were excluded from further analysis. Samples from DWTP 16 were not analyzed using the in vitro assay due to a limited amount of sample extract available.

2.2.3. In vitro data analysis

Data analysis was performed using GraphPad Prism (v5.0, San Diego, CA). Briefly, raw data were normalized to background, $\log_{10}$ transformed, and converted to % maximum response based on saturating levels of E2. Specifically, luminescence (RLU) for each well was normalized to the mean vehicle control (i.e., fold induction = well RLU/mean vehicle RLU) and then $\log_{10}$ transformed to correct for heterogeneity of variance (log fold induction). E2 standard concentrations (molar; x-axis) were then $\log_{10}$ transformed and plotted against the log fold induction (y-axis) and fit with a four-parameter logistic regression (bottom of curve constrained to 0) to determine the maximum E2 log fold induction (i.e., top of regression curve). Log fold induction values for each sample well were then converted to percent of maximum E2 response (%E2 max) by dividing by the top of the E2 standard curve and multiplying by 100. Next, sample concentrations (x-axis) were assigned according to the dilution factor from the assay (i.e., 1:1000 dilution = $1e^{-3}$ M, 1:2000 = $5e^{-4}$ M, 1:4000 = $2.5e^{-4}$ M, etc.). Sample concentrations were then log transformed and plotted as a function of %E2 max values and fit with a four-parameter logistic regression using the following constraints: top = 100, bottom = 0, and hillslope = hillslope of concurrent E2 standard curve. Finally, the E2 standard and sample EC_{50} values were determined from the logistic regression curves and used to calculate the estradiol equivalent (E2Eq) concentration of the sample using the following equation:

$$\text{E2Eq} = \text{E2 EC}_{50}/(\text{Sample EC}_{50})(\text{Sample pre} - \text{concentration factor})$$

where all concentrations are expressed in ng L^{-1} and the sample pre-concentration factor represents the degree to which the raw field sample was concentrated via solid phase extraction prior to testing. Specifically, source water samples from DWTP 1, 2, 10, 11, 15, 17, 18, 19, 20, 24, 25 were concentrated 2000-fold, while source water samples from DWTP 3, 4, 5, 12, 13, 14, 21, 22, 23, 26, 27, 28, 29 and all treated water samples were concentrated 4000-fold.

2.2.4. In vitro QA/QC

Bioassay sensitivity was determined using the minimum detectable concentration (MDC) as described by O'Connell et al. (1993) for use with bioassay data (Fig. 1). Briefly, data from all E2 standard curves in the study (n = 133 plates) were plotted as log concentration versus %E2 max response and fit using four-parameter logistic regression with bottom constrained to 0 and upper and lower 95% prediction intervals (PI) estimated using GraphPad Prism software. The MDC (0.1 ng E2Eq L^{-1}) for the E2 curve was defined as the concentration from the best fit line that was equal to the upper 95% PI for a zero (blank) response. At this concentration the expected response was greater than the variance of the blank response. The assay MDC was then further refined into sample reporting limits by taking into account the pre-concentration factors of the sample extracts. Specifically, the MDC of 0.1 ng E2Eq L^{-1} was multiplied by the bioassay dilution factor (1000-fold) and then divided by the sample pre-concentration factor (2000 or 4000-fold) resulting in sample reporting limits of 0.05 and 0.025 ng E2Eq L^{-1}, respectively.

Similar to the chemical analyses, in vitro assay analyses included primary and duplicate samples, blanks, and sample spikes (817 ng E2 L^{-1}). Analysis of spiked samples resulted in detection of 142 ± 42% (mean ± s.d.) of the spiked concentration (n = 49). There were no detections of estrogenicity above the reporting limits in any blank samples (n = 58).

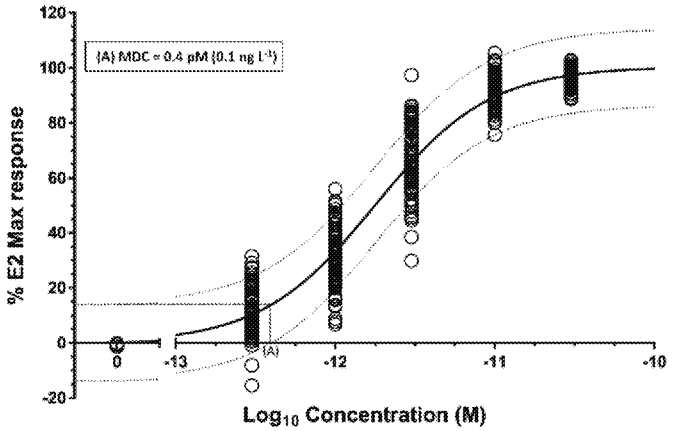

Fig. 1. Determination of minimum detectable concentration (MDC) in the T47D-KBluc transcriptional activation estrogenicity assay. Data from all E2 standard curves (n = 133) were plotted and fit with a four-parameter logistic regression and 95% prediction intervals (dotted lines). Ethanol vehicle controls were assigned a concentration of 0 M for graphical visualization. The MDC (0.1 ng E2Eq L^{-1}) was defined as the concentration at which the upper 95% PI of the blank meets the best fit line (A). See Materials & methods for further description of assay reporting limits.

Further, there were no significant differences in mean E2Eq between primary and duplicate field samples (*t*-test, p ≥ 0.05 for all comparisons).

3. Results

All estrogen concentrations (E1, E2, EE2, and E3) were below LC-FTMS quantitation limits in 20 of 25 (80%) source water samples and all treated water samples. E2, EE2, or E3 were not detected in any sample above their respective LCMRLs, however five source water samples (DWTP 3, 4, 16, 18, 25) had quantifiable concentrations of E1 (Fig. 2a) with concentrations ranging from 0.11–0.29 ng L^{-1}. Following DWTP processes, there were no detectable levels of E1 in treated water in any sample. Further, although not detailed here, there were no detections of additional known estrogen active compounds (e.g., bisphenol A, tamoxifen, 4-n-octylphenol, para-nonylphenol) above their respective LCMRLs in source or treated water (see Glassmeyer et al. (2017) for comprehensive list of analytes).

In contrast, in vitro estrogenicity was detected above the reporting limits in most source water samples (16 of 24) and a few treated water samples (3 of 24; Fig. 2b,c). Source water estrogenicity concentrations ranged from 0.044–0.47 ng E2Eq L^{-1} (mean, 0.19 ± 0.13 ng E2Eq L^{-1}), whereas treated water estrogenicity was significantly lower (*t*-test, p < 0.001), ranging from 0.037–0.078 ng E2Eq L^{-1} (mean, 0.054 ± 0.022 ng E2Eq L^{-1}). In general, there was good agreement in E2Eq values between replicate runs of a given sample with an average coefficient of variation of 18 ± 14% across all samples. Treated water samples, which were collected with DWTP residence time accounted for, indicated that in most cases (14 of 17) DWTP processes reduced the estrogenic activity found in the source water to below reporting limits. At the remaining sample sites, estrogenicity was either largely reduced (DWTP 13, 84% reduction) or moderately reduced (DWTP 1 and 23, 43 and 39% reduction, respectively). Overall, the utility of the assay was demonstrated by the positive detection of estrogenicity in 12 source and 3 treated samples for which there were no analytical detections of any natural or synthetic estrogenic compounds.

One of the goals of the study was to determine if there was a significant relationship between sample chemistry concentrations of estrogens and the in vitro bioassay results. A rigorous analysis was precluded by the fact that very few estrogen concentrations were detected above the LCMRLs with the analytical methods. However, the samples with the greatest E1 concentrations (DWTP 3, 4, 18) displayed

the greatest in vitro estrogenicity (Fig. 2a,b). After correcting for the potency of E1 in the T47D-KBluc assay (1.4-fold more potent than E2 (Bermudez et al., 2012)), we determined that in three of four source water samples (DWTP 3, 4, and 18) the anticipated response to E1 could account for 65–98% of the in vitro estrogenicity (Table 2). The exception was source water from DWTP 25 which displayed an E2Eq below (~2-fold lower) what was predicted based on the E1 concentration reported. Complete information on the full chemical analyses in addition to the estrogen concentrations reported here can be found in the overview manuscript by Glassmeyer et al. (2017).

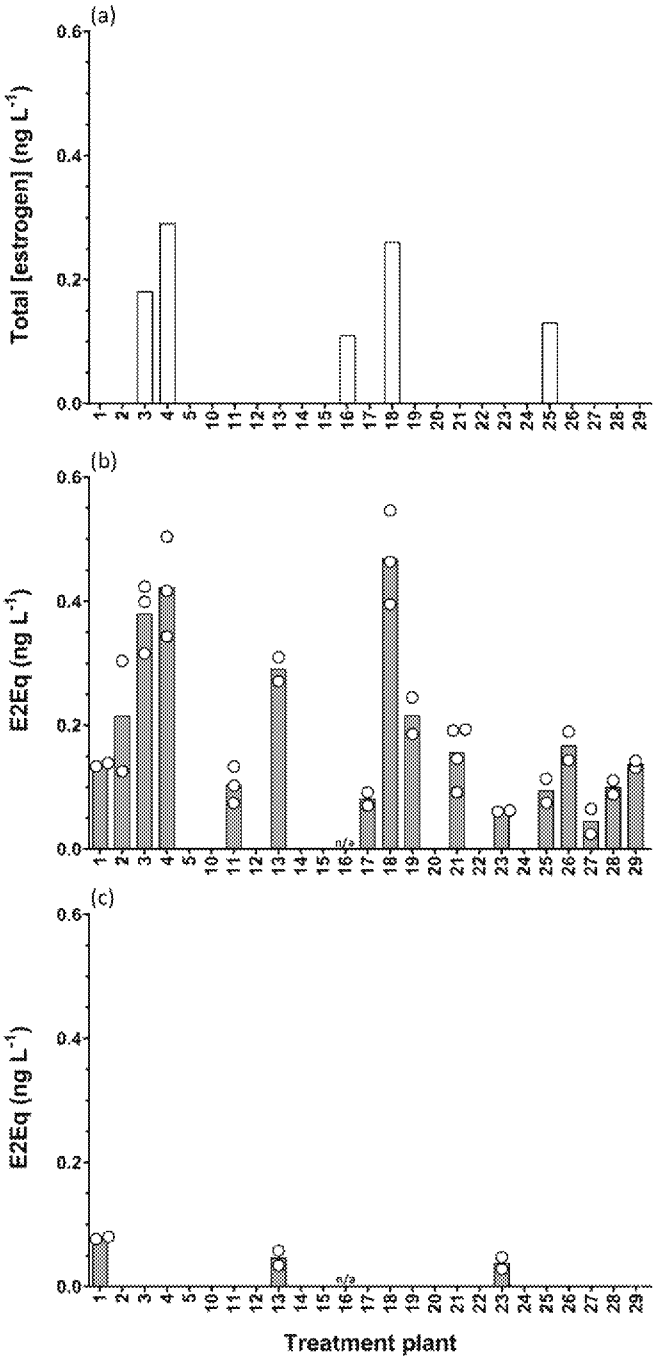

Fig. 2. Total concentration of estrogens in source water (a) and in vitro estrogenicity of source water (b) and treated water (c) from 25 US drinking water treatment plants using LC-FTMS and T47D-KBluc assay, respectively. Total estrogen concentration represents the concentration of E1 as it was the only estrogen detected above LCMRL (no detections of E2, EE2, or E3). Gray bars (panels (b), (c)) represent the mean E2Eq concentration for a given sample and white circles represent individual values for replicate runs of the sample in the assay. In vitro estrogenicity was not assayed for treatment plant 16.

4. Discussion

Several studies have reported the presence of steroidal estrogens in surface water samples similar to the source waters analyzed here. In most cases the predominant, or only, estrogen detected was E1 (Alvarez et al., 2013; Benotti et al., 2009; Esteban et al., 2014; Henneberg et al., 2014). Alvarez et al. (2013) detected E1 residues as high as 8.3 ng L^{-1} in stream samples associated with livestock operations in the USA and Esteban et al. (2014) reported concentrations up to 17 ng L^{-1} in Spanish surface waters. However, the bulk of reported E1 concentrations tend to be <1 ng L^{-1} (Benotti et al. (2009), ≤0.9 ng L^{-1}; Henneberg et al. (2014), ≤0.8 ng L^{-1}), similar to those found here. E1 is often the most frequently detected steroidal estrogen because it is excreted by humans and livestock at relatively high concentrations as compared to other estrogens (Wise et al., 2011) and excreted E2 is readily oxidized to E1 in surface waters (Jurgens et al., 2002) and during sewage treatment plant (STP) processes (Ternes et al., 1999). Once E1 enters a STP, or is converted from E2, it is relatively stable prior to discharge into surface water (Ternes et al., 1999). Work has been conducted on the isolated exposure of aquatic organisms to E1 (predominantly in fish) with effects typically reported from exposure to >10 ng L^{-1} (Dammann et al., 2011; Ghekiere et al., 2006; Imai et al., 2007; Panter et al., 1998; Van den Belt et al., 2004). Recently, Caldwell et al. (2012) calculated a predicted no effect concentration (PNEC) of 6 ng L^{-1} which is 20–54 -fold greater than all of the source water samples analyzed here. As such, the concentrations of E1 quantified in the present study do not appear to pose a significant risk to aquatic organisms.

In contrast to source/surface water, very few studies have reported detectable concentrations of natural estrogens, or EE2, in treated water from DWTPs. Metcalfe et al. (2014) recently reported concentrations of E1 (≤1.5 ng L^{-1}) in samples of treated water from Ontario. Kuch and Ballschmiter (2001) also reported detections of E1 (along with E2 and EE2) in German tap water, however these samples were from groundwater wells as opposed to DWTPs. Overall, it appears that in the majority of cases DWTP processes are adequate at reducing the concentrations of estrogens (notably E1) to levels below analytical detection limits, similar to the data reported here (Lee et al., 2008; Metcalfe et al., 2014; Ternes et al., 1999; Westerhoff et al., 2005).

Previous laboratory studies have reported reductions in the concentrations of E1, E2, EE2, and E3 (and the estrogenic activities associated with them) following reaction with chlorine (Cl_2) and ozone (O_3) in natural and model waters (Hu et al., 2003; Lee et al., 2008; Liu et al., 2005; Schenck et al., 2012; Westerhoff et al., 2005). In the present study E1 was quantified in source water, but not treated water, from DWTPs 3, 4, 16, 18, and 25. DWTPs 3 and 4 used Cl_2 as a disinfectant, DWTPs 18 and 25 used O_3 prior to chloramination, while DWTP 16 used chloramination. The reductions in the concentrations of E1 following disinfection reported here are consistent with those reported by Westerhoff et al. (2005) in which E1 reductions of 99% were observed

Table 2
Estimated proportional estrogenicity of estrone (E1) in source water samples containing both detectable E1 concentrations ([E1]) and in vitro estrogenicity (E2Eq).

DWTP	Total E2Eq (ng L^{-1})	[E1] (ng L^{-1})	E1 potency[a]	E1 E2Eq[b] (ng L^{-1})	E1 E2Eq[c] (% of total E2Eq)
3	0.38	0.18	1.4	0.25	65
4	0.42	0.29	1.4	0.41	98
18	0.47	0.26	1.4	0.36	77
25	0.094	0.13	1.4	0.18	191

[a] E1 potency in T47D-KBluc assay = E2 EC_{50}/E1 EC_{50} (from Bermudez et al., 2012).
[b] E1 E2Eq = [E1] * E1 potency.
[c] E1% of total E2Eq = (E1 E2Eq/total E2Eq) * 100.

following chlorination and ozonation. Monochloramine (NH_2Cl) is less reactive than O_3 or Cl_2 (Lee et al., 2008; Singer and Reckhow, 2011). However, in the USA, NH_2Cl is usually formed by addition of Cl_2 followed by ammonia. Consequently, E1 in a source water would be in contact with free Cl_2 for some period of time. Further, more than half of the treatment plants in this study used granular activated carbon (GAC) or powdered activated carbon (PAC) to remove organic contaminants. Adsorption on PAC has been reported to remove several estrogens, including E1, E2, EE2, E3 and bisphenol A (Schenck et al., 2012; Westerhoff et al., 2005; Yoon et al., 2003). However, adsorption efficiency varies widely depending on a number of parameters including water quality, carbon type/dosage, and compound hydrophobicity. Therefore, although some reduction of estrogenic compounds from the source waters would be expected due to carbon adsorption, the degree of reduction would likely be treatment plant specific.

The reductions in in vitro estrogenic activity observed in the present study were similar to the reductions in E1 concentrations, described above, mainly due to the disinfection procedures used by the DWTPs. Westerhoff et al. (2005) reported the oxidation of a large number of organic compounds, in addition to E1, following reaction with O_3 and/or Cl_2. Plants 1, 11, 18, 20, 22, 25, and 28 used O_3 as a pre-disinfectant followed by either Cl_2 or NH_2Cl. More than half of the treatment plants used Cl_2 as either a pre-disinfectant, final disinfectant, or both. The remaining plants (10, 19, 24 and 27) used NH_2Cl, which likely included some contact time with free Cl_2 as discussed above. The reaction of O_3 and Cl_2 with unidentified estrogenic compounds in the source waters likely accounts for at least part of the reductions in estrogenic activity following treatment observed in this study. A possible explanation for the estrogenic activity in three of the treated waters (DWTP 1, 13, and 23) could be the presence of unidentified estrogenic compounds that do not react readily with any of the disinfectants used at the individual plants. However, we cannot speculate on what those compounds could be nor was there a noticeable trend in treatment processes among those plants that may have contributed to the minimal estrogenic activity persisting into treated water.

There has been a recent increase in the use of bioassays to determine estrogenic activity (and other hormonal activities) in various water samples. The in vitro estrogenicity of DWTP source water samples analyzed here were similar to other studies of surface waters. Henneberg et al. (2014) reported ≤0.8 ng E2Eq L^{-1} downstream of WWTP effluent points using the E-Screen assay. Alvarez et al. (2013) reported ≤4.5 ng E2Eq L^{-1} using the T47D-KBluc assay from stream water associated with dairy cattle runoff, however ~87% of samples (streams impacted by dairy, swine, and poultry practices) were ≤1 ng E2Eq L^{-1}. Cavallin et al. (2014) reported 0.13–4.72 ng E2Eq L^{-1} in surface water collected from livestock farming operation basins using the T47D-KBluc assay. In some of these field studies estrogenic activity values exceeded current estimated trigger levels predicted to result in adverse effects in fish and other aquatic organisms. A de facto threshold of 1 ng E2Eq L^{-1} is frequently used for adverse effects on wild fish populations (Young et al., 2004). Similarly, Caldwell et al. (2012) calculated an E2 PNEC of 2 ng L^{-1} for effects on aquatic organisms. However, none of the source samples assayed here displayed E2Eq values above reported thresholds estimated to pose a risk to aquatic organisms.

A limited number of studies have recently used in vitro assays to screen for estrogenicity in treated drinking water. Brand et al. (2013) used ER CALUX bioassays to test Dutch drinking water and detected 0.022–0.032 ng E2Eq L^{-1}. Maggioni et al. (2013) used ERα transfected HeLa cells and determined a max of 0.0136 ng E2Eq L^{-1} in Italian drinking water. Overall, reported estrogenicity in treated drinking water tends to be ≤0.1 ng E2Eq L^{-1}, which is below the level predicted to result in adverse human health effects. The WHO (2000) recommended an acceptable daily intake (ADI) of 50 ng kg^{-1} day^{-1} (E2 equivalent) for humans, including a 100-fold safety factor and below which human health effects are not expected. For comparison, in the present study, assuming complete absorption, a person would have to consume ~38,360 L of the treated water sample with the greatest estrogenicity (0.078 ng E2Eq L^{-1}) to reach the ADI. In addition, significantly higher levels of naturally occurring estrogens have been found in milk and other foods than in drinking water (Caldwell et al., 2010). Overall, the levels of estrogenic activity in treated water samples found here do not appear to pose a significant human health risk.

One important factor in the use of in vitro bioassays that often receives only cursory description is a clear determination of the assay sensitivity limit. Commonly, the limit of detection (LOD) is represented by the concentration that is equal to three times the standard deviation of replicate blank runs. However, this approach is less appropriate when applied to typical bioassay results because the instrumental response data (RLUs) are normalized to the corresponding vehicle blank response in order to correct for plate-to-plate variability in cellular activity. As such, the %E2 max response for each blank replicate is essentially zero and has a small standard deviation (%E2 max response mean ± s.d., −0.56 ± 0.98) resulting in an artificially low LOD (e.g., 0.02 ng E2Eq L^{-1}; 0.005 or 0.01 ng L^{-1} reporting limits after correcting for sample pre-concentration of present data). Based on visual inspection of the data and statistical analyses of individual sample dilutions, the reporting limits calculated from the traditional LOD approach were determined to be unacceptably low due to a lack of induction of ER activation in the T47D-KBluc assay. Instead, we chose to use the MDC as described by O'Connell et al. (1993) to determine the reporting limits in this study and recommend this method in future studies. At the 95% MDC there was a ~5% chance of a type I error (false positive), however, increasing confidence (99% prediction interval) resulted in a minimal increase in MDC (0.11 versus 0.14 ng L^{-1}). Therefore, we utilized 0.1 ng L^{-1} as a defensible minimum detectable concentration for determination of reporting limits that were sensitive to positive detections without being overly conservative against type I errors.

Another consideration in the use and reporting of in vitro bioassay data has been the translation from in vitro activity/potency to in vivo potency/effects (Henneberg et al., 2014). Each of the endogenous and synthetic steroid hormones display disparate potencies across the gamut of commonly used assays (i.e., T47D-KBluc, E-Screen, ER-CALUX, YES, MELN, MVLN) (Jarosova et al., 2014). In vivo, the estrogens have widely different half-lives, serum protein binding, tissue distribution, metabolic pathways, and bioaccumulation potential, which are factors the in vitro assays do not account for. Similarly, each of those compounds display disparate activities across aquatic and terrestrial test species, and among different tissues and life stages. For these reasons, the detection of in vitro estrogenic activity within a given water sample cannot necessarily be directly extrapolated to adverse in vivo effects. However, in vitro bioassay data can be used as an important screening step in toxicological assessment to guide additional in vivo screening and testing (Cavallin et al., 2014). Therefore, combining in vitro and in vivo bioassays and chemical analyses of estrogenicity provides a considerable advantage as opposed to using only one method.

5. Conclusions

The value of adding bioanalytical tools to augment chemical analyses is demonstrated in the present study. The in vitro assays successfully identified samples that contained detectable levels of E1 as well as twelve samples which had estrogenic activity that was not accounted for in the chemical analyses. In total, 67% of the source water samples analyzed had E2Eq values above the reporting limits, whereas only 20% had quantifiable concentrations of individual steroidal estrogens. To illustrate this point, if a hypothetical water sample contained each of the four estrogens at concentrations 10-fold below their respective LCMRLs the T47D-KBluc assay would theoretically detect an E2Eq of ~0.31 ng L^{-1} (i.e., 5–10-fold greater than the assay reporting limits). It is also possible that non-detected compounds were present in the

samples that contributed to the observed estrogenic activity. Thus, selective analytical chemical determinations suffer from the identification/quantification of only pre-selected target analytes and the inability to identify interactions of toxicant mixtures (additivity, synergism, antagonism, etc.) (Waller et al., 1996), both of which can be compensated for by the use of in vitro bioassays.

Despite their own limitations, in vitro bioassays clearly have utility as screening tools in water quality monitoring and toxicological assessment. Applying bioactivity measures to water quality monitoring has the potential to permit more comprehensive evaluation of water quality efficiently and effectively and guide further testing and assessment. However, extensive use of in vitro bioassays will require development of effective techniques adapted to a variety of media, and a change in the regulatory construct to one that is not focused on single chemical measures.

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